Supplemental Information

Genetic identification of mechanoinsensitive 'silent' nociceptors.

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Supplemental Figures

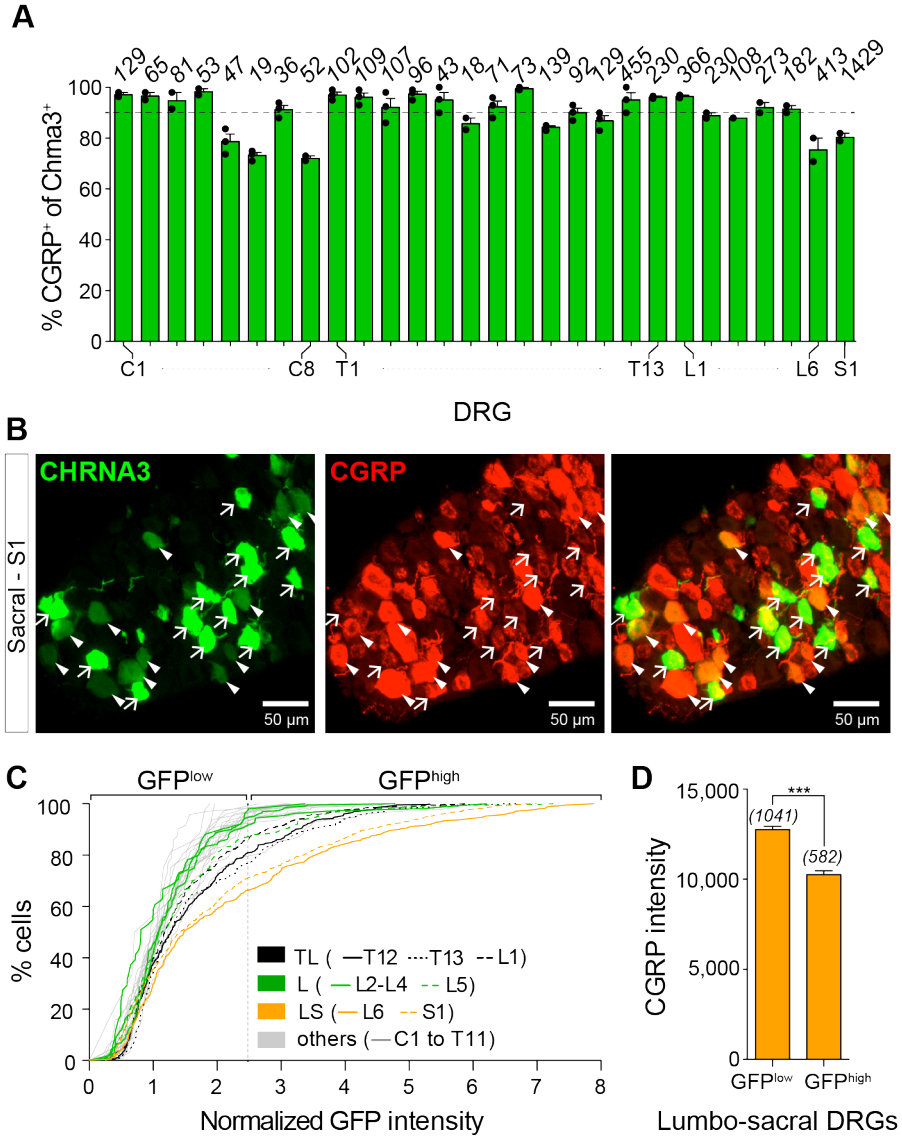

Figure S1, related to Figure 2, Lumbosacral DRGs comprise two populations of CHRNA3$^+$ neurons

(A) Bar graph showing the percentage of CHRNA3$^+$ neurons that express CGRP. Bars represent means ± s.e.m. (N = 2 - 3 DRGs from 2 - 3 mice) and individual percentages of each analyzed DRG are shown as black circles. Numbers above the bars represent the total number of analyzed CHRNA3$^+$ neurons.

(B) Representative images showing CGRP expression in GFP^{low} (arrowheads) and GFP^{high} (arrows) CHRNA3$^+$ neurons.

(C) GFP intensities from individual DRGs and different experiments were fitted with a Gaussian function and with the sum of two Gaussians. The best fitting model was determined using the extra sum-of-squares F test of Graphpad Prism 5. C1 - T11 and L2 - L5 GFP intensity distributions were best fitted with a single Gaussian and with the sum of two Gaussians for T12 - L1 and L6 - S1 DRGs. The graph shows the cumulative distribution of GFP intensities normalized to the mean intensity value of the GFP^{low} population of the indicated DRGs.

(D) Bar graph showing that GFP^{low} neurons express higher levels of CGRP. Bars represent means ± s.e.m.. The numbers of analyzed cells are indicated above the bars. ***, P<0.001, Student's T-test.

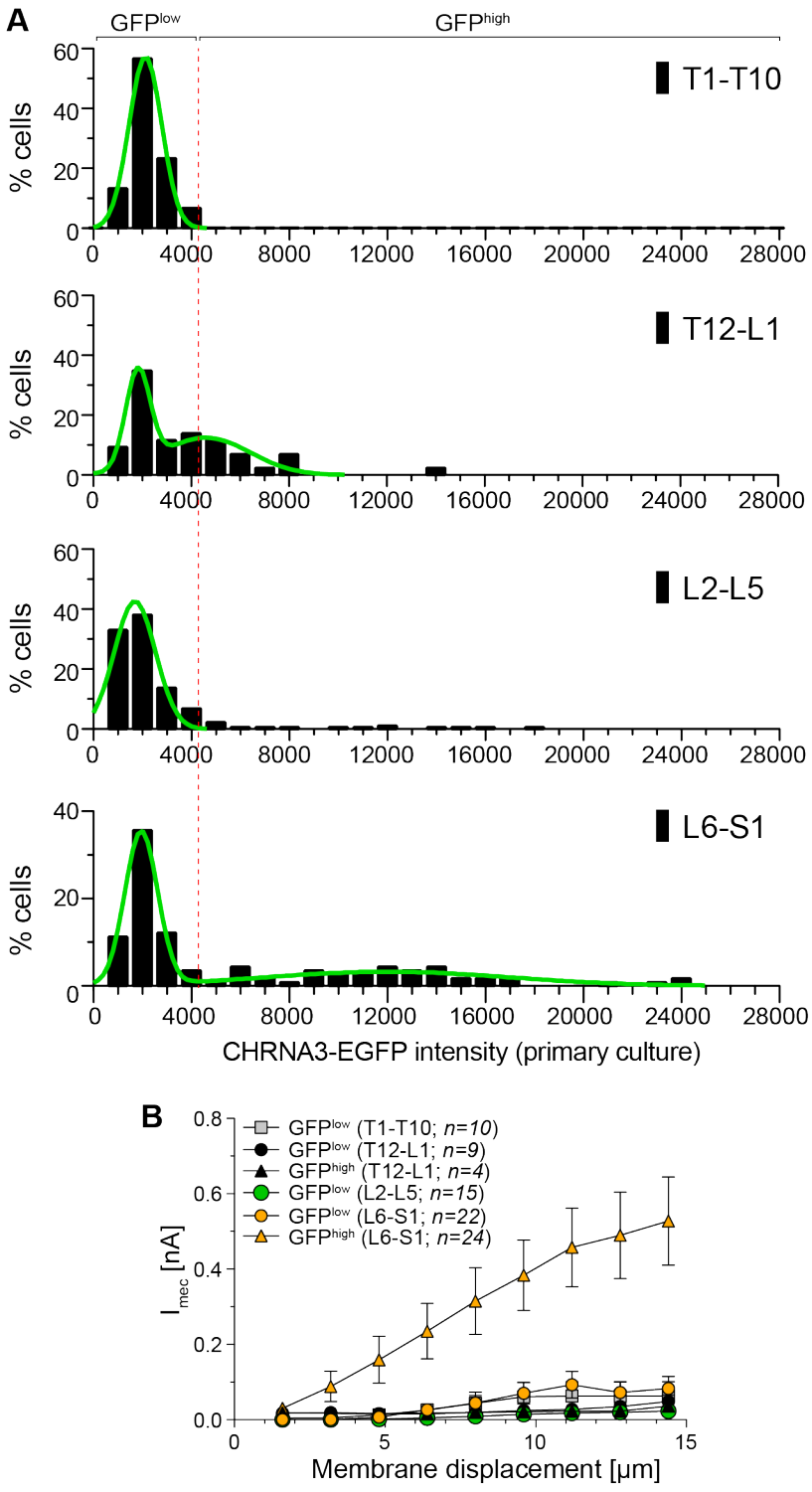

Figure S2, related to Figure 2, GFP^{low} and GFP^{high} CHRNA3^{+} neurons have different functional properties.

(A) Frequency distribution plot of GFP-intensities showing that GFP^{low} and GFP^{high} neurons can also be distinguished by means of the endogenous GFP signals in DRG cultures. The 90th percentile of the L2-L5 GFP-intensity distribution was used as the cut-off to distinguish between GFP^{low} and GFP^{high} neurons (vertical dashed line).

(B) Mean ± s.e.m. amplitudes of mechanically evoked currents in CHRNA3^{+} GFP^{low} and GFP^{high} neurons from the indicated spinal segments are shown as a function of membrane displacement. Note that only GFP^{high} neurons from L6-S1 exhibit mechanotransduction currents.

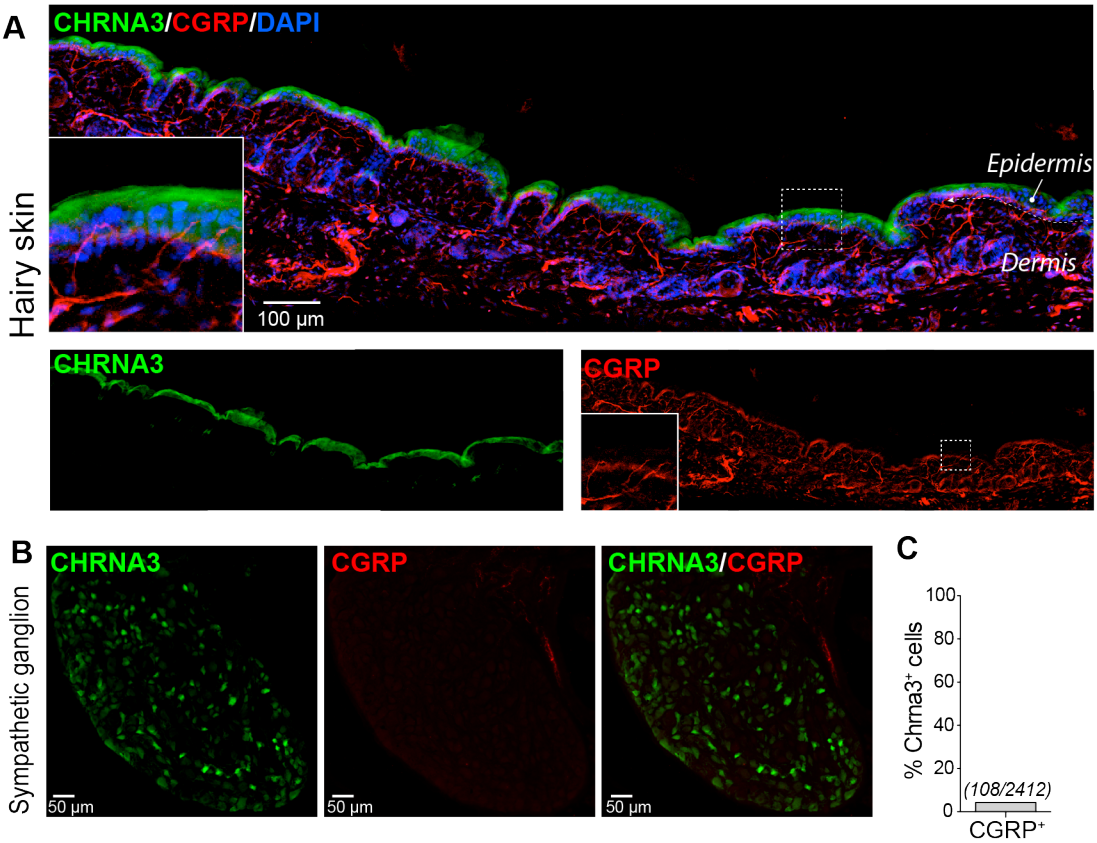

Figure S3, related to Figure 3, CHRNA3$^+$ afferents do not innervate hairy skin

(A) Immunostainings showing that CHRNA3$^+$ afferents do not innervate the hairy skin.

(B) Immunostaining showing that some neurons in sympathetic ganglia also express CHRNA3; these neurons do however not express CGRP.

(C) Bar graph showing the percentage of CHRNA3$^+$ sympathetic neurons that express CGRP.

Supplemental Experimental Procedures

Animals

CHRNA3-EGFP mice, official name Tg(Chrna3-EGFP)BZ135Gsat/Mmnc (RRID:MMRRC_000243-UNC) were obtained from the Mutant Mouse Resource & Research Center (MMRRC) and were backcrossed to a C57Bl/6J background. Mice were housed in the Interfacultary Biomedical Facility of Heidelberg University according to institutional guidelines. All animal experiments were carried out according to the German Animal Protection Law and with permission of the Regierungspraesidium Karlsruhe (T-57/16). Retrograde tracing experiments were conducted at the University of Cambridge in accordance with the United Kingdom Animal (Scientific Procedures) Act 1986 Amendment Regulations 2012 under a Project License (70/7705) granted to E. St. J. S. by the Home Office; the University of Cambridge Animal Welfare Ethical Review Body also approved procedures.

Immnuohistochemistry

DRGs were dissected in ice-cooled PBS, fixed with 4% PFA for 30 min at 4 °C and incubated overnight in 30 % sucrose at 4°C. DRGs were then embedded in Tissue-Tek O.C.T compound and cut into 16 µm cryo-sections. After drying, sections were incubated in 50 mM Glycine for 20 min, washed twice with PBST (0.2 %), blocked with PBST (0.2 %) + 10% donkey serum + 1% BSA and then incubated with primary antibodies for 1 h at room temperature. Primary antibodies were diluted in PBST (0.2 %) + 10 % donkey serum. Sections were then washed four times with PBST (0.2 %), subsequently incubated with secondary antibodies for 1 h at RT, washed with PBST four times, dried and mounted with fluorogel (Fluoprobes).

Glabrous skin, hairy skin, urinary bladder, distal colon, gastrocnemius muscle and spinal cord samples were dissected in cold PBS and fixed with Zambonis fixative for 2 h at RT, washed four times and incubated in 30 % sucrose at 4 °C overnight. Knee joints were fixed with Zambonis fixative over night. Prior to cutting, knee joints were decalcified by submerging the samples in PBS + 10 % EDTA for 7 days (PBS/EDTA was replaced every day). For the preparation of tissue sections, samples were embedded in Tissue-Tek, frozen with liquid nitrogen and cut into 50 µm cryo-sections. After drying, sections were incubated in 50 mM Glycine for 45 min, washed twice with PBST (0.2 %), blocked 1 h with PBST (0.2 %) + 10 % donkey serum + 1 % BSA and then incubated with primary antibodies overnight at 4°C. Primary antibodies were diluted in PBST (0.2 %) + 10 % donkey serum + 1 % BSA. Sections were then washed several times with PBST (0.2 %), subsequently incubated for 4 hours with

secondary antibodies in PBST (0.2 %) + 10% donkey serum + 1% BSA at room temperature, washed with PBST (0.2%) several times, dried and mounted with fluorogel (Fluoprobes).

Antibodies

The following primary antibodies were used: rat anti-GFP (Nacalai tesque, #04404-84, 1:3000; RRID:AB_10013361), mouse anti-Nefh (Sigma-Aldrich, N0142, 1:600, RRID:AB_477257), goat anti-TrkA (R&D, AF1056, 1:200, RRID:AB_2283049), rabbit anti-CGRP (ImmunoStar, 1:200, RRID:AB_572217), rabbit anti-PIEZO2 (Novus, 1:100, RRID:AB_11008402) (Florez-Paz et al., 2016; Narayanan et al., 2016), and Isolectin GS-IB4-Alexa Fluor® 568 Conjugate (Life technologies, 3 µg/ml). Secondary antibodies were AlexaFluor-488 donkey anti-rat (Life technologies, AF21208, 1:500, RRID:AB_2535794), AlexaFluor-594 donkey anti-mouse (Life technologies, AF21203 1:500, RRID:AB_2535789), AlexaFluor-633 donkey anti-goat (Life technologies, A21082, 1:500, RRID:AB_10562400), AlexaFluor-594 donkey anti-rabbit (Life technologies, A2107, 1:500, RRID:AB_141637).

Cell culture

8-12 weeks old mice were killed by placing them in a CO_2-filled chamber for 2–4 min followed by cervical dislocation and DRGs were collected in Ca^{2+} and Mg^{2+}-free PBS. DRGs were subsequently treated with collagenase IV for 30 minutes (1 mg/ml, Sigma) and with trypsin (0.05 %, Life Technologies) for a further 30 minutes, at 37 °C. Digested DRG's were washed twice with growth medium [DMEM-F12 (Invitrogen) supplemented with L-glutamine (2 µM, Sigma), glucose (8 mg/ml, Sigma), penicillin (200 U/ml)–streptomycin (200 µg/ml) (both Life Technologies) 5 % fetal horse serum (Life Technologies)], triturated using fire-polished Pasteur pipettes and plated in a droplet of growth medium on a glass coverslip precoated with poly-L-lysine (20 µg/cm², Sigma) and laminin (4µg/cm², Life Technologies). To allow neurons to adhere, coverslips were kept for 3 - 4 hours at 37 °C in a humidified 5 % incubator before being flooded with fresh growth medium. Cultures were used for patch-clamp experiments on the next day.

Patch-clamp recordings

Whole cell patch clamp recordings were made at room temperature (20-24°C). Patch pipettes with a tip resistance of 2-4 MΩ were pulled (Flaming-Brown puller, Sutter Instruments, Novato, CA, USA) from borosilicate glass capillaries (BF150-86-10, Sutter Instrument), filled with a solution consisting of 110 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM HEPES, 2 mM guanosine 5'-triphosphate (GTP) and 2 mM adenosine 5'-triphosphate (ATP) adjusted to pH 7.3 with KOH. The bathing solution contained 140 mM NaCl, 4 mM

KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 4 mM glucose, 10 mM HEPES, adjusted to pH 7.4 with NaOH. Drugs were applied with a gravity driven multi-barrel perfusion system (Valvelink8.2, Automate Scientific). All recordings were made using an EPC-10 amplifier (HEKA, Lambrecht, Germany) in combination with Patchmaster© and Fitmaster© software (HEKA). Pipette and membrane capacitance were compensated using the auto function of Patchmaster and series resistance was compensated by 70 % to minimize voltage errors.

Mechanically activated currents were recorded in the whole-cell patch-clamp configuration. Neurons were clamped to a holding potential of -60 mV and stimulated with a series of mechanical stimuli in 1.6 µm increments with a fire-polished glass pipette (tip diameter 2-3µm) that was positioned at an angle of 45° to the surface of the dish and moved with a velocity of 3.5µm/ms by a piezo based micromanipulator called nanomotor© (MM3A, Kleindiek Nanotechnik, Reutlingen, Germany). The evoked whole cell currents were recorded with a sampling frequency of 200 kHz. Mechanotransduction current inactivation was fitted with a single exponential function ($C_1+C_2*\exp(-(t-t_0)/\tau_{inact})$, where C1 and C2 are constants, t is time and τ_{inact} is the inactivation time constant. Currents with a $\tau_{inact} < 10$ ms were classified as RA-type currents, currents with τ_{inact} between 10 and 50 ms as IA-type currents and currents that with $\tau_{inact} > 50$ ms as SA-type currents (Lechner and Lewin, 2009). For classification of sensory neurons, action potentials were recorded in current-clamp mode and evoked by repetitive 80 ms current injections increasing from 40 pA to 800 pA in increments of 40 pA.

Retrograde labeling

Retrograde labeling of sensory neurons innervating the knee was performed in CHRNA3-EGFP (male, 11-15 weeks old) mice as described previously (da Silva Serra et al., 2016) using Fast Blue (FB; 2 % in saline, Polysciences Gmbh, Germany). Mice were anaesthetized by an intra-peritoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Once no withdrawal reflexes were observed, mice (n = 4) received FB intraarticular injections in both hind limb knees (1.5 µl). Injections were performed using a 10 µl Hamilton syringe and a 30G needle. Retrograde labeling of sensory neurons innervating visceral organs (bladder and colon) was also performed in CHRNA3-EGFP mice (male, 11-15 weeks old) using FB as previously described (Hockley et al., 2014, 2016, 2017). In brief, mice were anaesthetized using isofluorane (4 % induction / 1.5 % maintenance) and a mid-line laparotomy performed to reveal the visceral organs. FB was injected into the wall of the distal colon (n = 3, 6 injections per animal, total volume ~2 µl), and in separate animals, the bladder (n = 3, single injection per animal, ~5 µl), before closure of the laparotomy and allowing the animal to recover. Post-operative care (glucose-enriched soft diet) and analgesia (buprenorphine 0.05-0.1 mg/kg subcutaneously) was provided for 5 days. After 7 days, for knee-labeled mice,

and 14 days, for colon- and bladder-labeled mice, animals were anaesthetized with sodium pentobarbital (140 mg/kg, intraperitoneally) and transcardially perfused with saline (0.9 % NaCl) followed by 4 % PFA in PBS. Lumbar (L3-L4) DRG were dissected from knee-labeled mice and thoracolumbar (T13) and lumbosacral (S1) DRG were dissected from colon- and bladder-labelled mice. All DRG dissected were post-fixed for 30 mins in 4 % PFA, washed in PBS and cryoprotected in 30 % sucrose overnight.

Single cell electroporation and siRNA-mediated knockdown

CHRNA3$^+$ neurons were transfected with non-targeting siRNA (, D-001810-01-05 ON-TARGETplus Non-targeting siRNA, GE-Healthcare) and PIEZO2-siRNA (L-163012-00-0005 ON-TARGETplus Mouse Piezo2 (667742) siRNA – SMARTpool), respectively, using single cell electroporation (Bestman et al., 2006; Haas et al., 2001). 4 hours after plating, single CHRNA3$^+$ neurons were approached with 1.5 MOhm patch pipettes filled with 5 µM siGLO RED (GE-Healthcare) transfection indicator and 500 nM non-targeting siRNA or 500 nM PIEZO2-siRNA diluted in intracellular patch-clamp buffer (see above). The patch clamp electrode and the bath electrode were connected to an isolated pulse stimulator (A-M Systems, Model 2100). As soon as the patch pipette touched the cell surface, 2 consecutive trains of square shaped electrical pulses (amplitude = 2.5 V, pulse duration = 2 ms, frequency 200 Hz, train duration 500 ms) were applied to electroporate the membrane and to drive siGLO and siRNA into the cell. After transfection, cultures were kept in the presence of 50ng/ml NGF for 72 h at 37 °C in a humidified 5 % incubator prior to cell collection or patch clamp recordings. Patch-clamp recording from siRNA-transfected neurons were performed as described above. The efficiency of PIEZO2 knock-down was tested by qPCR. To this end samples of five transfected neurons were aspirated into the patch pipette and processed as described below.

Reverse transcription and quantitative real-time PCR

mRNA expression levels of candidate mechanotransduction genes in CHRNA3+ neurons were determined as follows. Samples containing 20 CHRNA3$^+$ neurons were collected from DRG cultures by aspirating the cells into a patch clamp pipette with a tip diameter of 25 µm, filled with 2-4µl PBS containing 4 U/µl RNAseOUT (Thermofisher). For each gene three to four samples (one sample per mouse from a total of 3 - 4 CHRNA3-EGFP mice) were collected. cDNA synthesis was carried out directly on the sample using the Power SYBR® Green Cells-to-CT™ Kit (Life Technologies) following the manufacturers instructions. qPCR reactions were set up using FastStart Essential DNA Green Master (Roche) by adding 4 µL of the obtained cDNA as template and the following primer pairs at a concentriation of 250 nM:

ASIC2_ FWD	5’-GGCTTACTGGCAGAAAAGGA-3’
ASIC2_ REV	5’-CTTGCTGGGGATCTTTACCA-3’
ASIC3_ FWD	5’-GAGACATTGGGGGACAGATG-3’
ASIC3_ REV	5’-CCCCAGGACTCTGTCTTGAA-3’
TRPA1_FWD	5’-CACAGACCGACTAGATGAAGAAGG-3’
TRPA1_REV	5’-GGGCAATATGCAGAAAGGAGG-3’
TRPC3_FWD	5’-GGAGAGCGATCTGAGCGAAGT-3’
TRPC3_REV	5’-GGGAGCCATTTGTCTCTAGCA-3’
TRPC6_ FWD	5’-ACTACATTGGCGCAAAACAGAA-3’
TRPC6_ REV	5’-AGAAAGACCAAAGATAGCCCAGAA-3’
TMEM150c_ FWD	5’-TAGCCCTCGTGGTAGCTGTT-3’
TMEM150c_ REV	5’-CATCGTTTGTGAGCTGGAAA-3’
PIEZO2_ FWD	5’-TTCAACCAGGGGTCCCAAGC-3’
PIEZO2_ REV	5’-TCCCAATTACAAGGACAACAGATGC-3’
GAPDH- FWD	5’-GCATGGCCTTCCGTGTTC-3’
GAPDH- REV	5’-GTAGCCCAAGATGCCCTTCA-3’

qPCR reactions were performed in a LightCycler 96 (Roche) with a thermal cycler profile as follows: 10 min preincubation step at 95°C followed by 40 cycles of PCR with a 10 second denaturing cycle at 95°C, followed by 10 seconds of annealing at 60ºC and 10 seconds extension at 72°C.

Supplemental References

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